On the Determination of Molecular Weight by Means of Small-Angle X-ray Scattering (Commemoration Issue Dedicated to Professor Ken-ichi Katayama On the Occasion of His Retirement)

AUTHOR(S):

Mimura, Mitsuru; Yamanaka, Shigenori; Urakawa, Hiroshi; Kajiwara, Kanji

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Mitsuru MIMURA*, Shigenori YAMANAKA*, Hiroshi URAKAWA*, Kanji KAJIWARA*

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Although synchrotron radiation provides a powerful X-ray source for scattering experiments, the absolute measurement of scattered X-ray intensity causes a practical problem. Since the conventional molecular weight determination by small-angle X-ray scattering requires the absolute intensity to be evaluated accurately, no experiment was undertaken so far to determine the molecular weight by synchrotron radiation small-angle X-ray scattering. A theory was developed to evaluate the molecular weight without knowledge of absolute scattered intensity, and the theory was examined by four biopolymers including lysozyme,bovine serum albumin and pullulan.

KEY WORDS: Synchrotron radiation/Small-angle X-ray scattering/Molecular weight determination/Lysozyme/Bovine serum albumin/Pullulan

INTRODUCTION

Synchrotron radiation provides a powerful source for X-ray, which is widely employed to investigate molecular structures in gas, liquid or solid[1]. A powerful X-ray is especially advantageous for scattering experiments from solutions, since excess X-ray scattered from solutes is so weak in intensity and requires several hours to measure with a reasonable signal-to-noise ratio when a conventional X-ray tube is employed for a source. In principle, the structure analysis based on the small-angle X-ray scattering yields the mass and size of colloidal particles between tens and several hundreds Å[2,3]. For example in X-ray scattering, the absolute scattered intensity $I_n(q)$ at the scattering angle 2θ is given in terms of the molecular weight M of colloidal particles as

$$I_n(q)=\frac{I_e\cdot\Delta z^2\cdot M\cdot d\cdot N_A\cdot c}{a^2}\cdot\phi(q) \tag{1}$$

where $I_e=7.9\times10^{-26}$[cm^2] is the Thomson factor representing a scattering intensity from a single electron, q the reduced scattering variable given by $q=(4\pi/\lambda)\cdot\sin\theta$, d [cm] the sample thickness, N_A the Avogadro number, c[g/cm^3] the concentration, a [cm] the distance between the sample and detector, and $\phi(q)$ the normalized form factor. Δz denotes the number of effective mole-electrons given by

$$\Delta z=z-\bar{v}\cdot z_0 \tag{2}$$

with z and z_0 being the the number of mole-electrons per gram particles and per cubic

* 三村 充, 山中 重宣, 浦川 宏, 梶原 莞爾: Faculty of Engineering & Design, Kyoto Institute of Technology, Kyoto, Sakyo-ku, Matsugasaki, 606 Japan

centimeter solvent, respectively, and $\bar{v}$ the specific partial volume of a particle. By definition $\phi(0)=1$, so that the molecular weight is determined from the scattered intensity at zero angle as

$$M=\frac{I_n(0)\cdot a^2}{\Delta z^2\cdot d\cdot c\cdot I_e\cdot N_A} \tag{3}$$

Eq. (3) can be rewritten in terms of the scattering amplitude b[cm/g] of a particle (given by the sum of coherent scattering lengths) and the scattering density ρ_0[cm^{-2}] of solvent as

$$M=\frac{N_A}{c(\Delta b)^2}\frac{a^2 I_n(0)}{d} \tag{4}$$

where the excess scattering density Δb[cm/g] is defined as

$$\Delta b=b-\bar{v}\rho_0, \tag{5}$$

and is related to the number of effective mole-electrons as

$$I_e\Delta z^2=(\Delta b/N_A)^2 \tag{6}$$

Eq. (4) requires the absolute scattered intensity and the excess scattering density to be measured with a reasonable accuracy in order to evaluate the molecular mass. Several techniques are proposed for the absolute measurement in X-ray scattering, although none can be applied satisfactorily to the synchrotron radiation X-ray source of extremely high intensity. Large error in evaluating the excess scattering density is involved through the partial specific volume $\bar{v}$, which should be measured with less than a 1% error to yield the molecular mass with a reasonable accuracy. Thus,the molecular mass determination by small-angle X-ray scattering is less practical, compared with other methods such as light scattering, osmotic pressure and sedimentation.

Since an initial slope of scattering profile is determined solely by the radius of gyration R_G, R_G can be evaluated without any knowledge of absolute scattered intensities as exemplified by the Guinier approximation

$$I_n(q)=I_n(0)\cdot\exp(-q^2R_G^2/3) \tag{7}$$

where the relative scattered intensity $I_n(q)/I_n(0)$ suffices to evaluate the radius of gyration. In practice, the radius of gyration is an only structure parameter evaluated from small-angle X-ray scattering, although the molecular mass can be estimated simultaneously.

The present report concerns with molecular weight determination without knowing the absolute scattered intensity. The theory is reviewed in the following section, and the method is evaluated with four biopolymers of molecular weight ranging from 5,000 to 66,000, with two being a compact protein and the rest a polysaccharide of random coil configuration.

THEORETICAL BACKGROUND

The mean square fluctuation of electron density $\overline{(\Delta\rho)^2}$[4] is given by

$$\frac{\overline{(\Delta\rho)^2}}{c}=\frac{1}{2\pi^2}\int_0^\infty \frac{a^2 I_n(q)}{d}\cdot q^2\cdot \mathrm{d}q \tag{8}$$

which reduces in the case of a two-phase system characterized by the scattering density ρ_0 and ρ_1 to

$$\overline{(\Delta\rho)^2}=V_0\cdot V_1\cdot(\rho_1-\rho_0)^2 \tag{9}$$

Here V_0 and V_1 represent the volume fractions of two phases, respectively, so that $V_1=1-V_0$. Considering the solution of homogeneous particles as a special case of a two-phase system, we may assume that eq.(9) is valid in a good approximation. Since the volume fraction is given in terms of concentration and partial specific volume as

$$V_1=c\bar{v}, \tag{10}$$

eqs. (5) and (9) yield

$$\frac{\overline{(\Delta\rho)^2}}{c}=\frac{1}{\bar{v}}\cdot(\Delta b)^2-c(\Delta b)^2 \tag{11}$$

where the scattering density ρ_1 of a particle is substituted by $b/\bar{v}$. Thus the initial slope of the $\overline{(\Delta\rho)^2}/c$ vs. c curve is identified to the square excess scattering amplitude as

$$\frac{\mathrm{d}}{\mathrm{d}c}\frac{\overline{(\Delta\rho)^2}}{c}=-(\Delta b)^2 \tag{12}$$

In general, the scattered intensity is considered as composed of two terms due to single particles and interparticular interferences, respectively:

$$I_n(q)\propto cP(q)+c^2Q(q) \tag{13}$$

where $P(q)$ and $Q(q)$ represent the scattering from single particles and interparticular interferences. The interference term in eq. (13) will be neglected when $c\ll 1$ and/or q is sufficiently high. That is, no interference effect will appear in scattering profile when q exceeds a certain finite value q_m which may depend on concentration.

To evaluate the mean square fluctuation of electron density, eq. (8) requires the value of scattered intensity $I_n(q)$ to be measured over a whole q range from 0 to ∞. Since no concentration dependence is expected in observed $I_n(q)$ when $q>q_m$, eq. (8) can be converted as

$$\frac{\mathrm{d}}{\mathrm{d}c}\frac{\overline{(\Delta\rho)^2}}{c}=\frac{1}{2\pi^2}\frac{\mathrm{d}}{\mathrm{d}c}\int_0^{q_m}\frac{a^2 I_n(q)}{cd}\cdot q^2\cdot \mathrm{d}q \tag{14}$$

where the integration limit q_m denotes the maximum q value above which no concen-

tration dependence will be observed in the scattering profile. Combining eqs. (12) and (14), the excess scattering density is given by

$$(\Delta b)^2=\frac{1}{2\pi^2}\frac{\mathrm{d}}{\mathrm{d}c}\int_0^{q_m}\frac{a^2I_n(q)}{cd}\cdot q^2\cdot \mathrm{d}q \tag{15}$$

which is inserted in eq.(4) to yield the molecular weight as

$$M=2\pi^2N_A\frac{a^2I_n(0)}{cd}\Big/\frac{\mathrm{d}}{\mathrm{d}c}\int_0^{q_m}\frac{a^2I_n(q)}{cd}\cdot q^2\cdot \mathrm{d}q \tag{16}$$

Here the term $I_n(q)$ appears in numerator and denominator,so that the term $I_n(q)$ in eq. (16) can be replaced by the scattered intensity $I(q)$ in an arbitrary unit.

MATERIALS AND METHODS

Materials

Commercially available proteins (lysozyme from chicken egg white and bovine serum albumin abbreviated as BSA purchased from Sigma) and polysaccharide for molecular weight calibration (pullulan purchased from Showa Denko) were employed

Table I Sample Characteristics.

Samples	Solution Concentration ($g \cdot cm^{-3}$)	Rg (Å)	Molecular Weight ($g \cdot mol^{-1}$)	
Lysozyme		15.2[9]	14,000[8]	SIGMA CHEMICAL CO.
	0.029812			
	0.041611			
	0.059958			
	0.124070			
BSA		29.8[7]	66,100[6]	SIGMA CHEMICAL CO.
	0.012370			
	0.036440			
	0.045912			
	0.068331			
	0.135880			
Pulullan P-5		22.9[10]	5,800*	Showa Denko K.K.
	0.026090			
	0.052950			
	0.103520			
P-10		35.0[10]	12,200*	
	0.014480			
	0.032510			
	0.087629			
	0.126650			
	0.580330			

The values of radius of gyration and molecular weight are taken or calculated from cited literatures.

* The molerular weight is specified by the supplier.

for present SAXS measurements. The characteristics of these samples are summarized in Table I. Solutions were prepared individually by weighing to each prescribed concentration, where phosphate buffer (pH 7.4) and bidistilled water were used for protein samples and pullulan as solvent, respectively. Those solutions were kept in a refrigerator till SAXS measurements.

Small-Angle X-ray Scattering

SAXS from polymer solutions were recorded through the optics and detector system of SAXES installed in the BL-10C of the Photon Factory, the National Laboratory for High Energy Physics, Tsukuba, Japan[5]. A wavelength λ of 0.149 nm was used and the specimen-to-detector distance was set to approximately 1900 mm. X-ray scattering was recorded at 512 channels of the one-dimensional position-sensitive proportional counter (an effective length 200 mm, Rigaku Denki Co.), ranging from $q=1.3\times10^{-1}$ to 3.35 nm^{-1} (equivalent to the Bragg spacing from $d_B=48.3$ to 1.87 nm). The solution temperature was controlled to 20 ± 0.1°C during the SAXS measurements. The counting time was 600 sec for each measurement, and the scattering magnitude was calibrated by using the diffraction peaks of collagen fiber. Excess scattering intensities were calculated by subtracting the scattering intensity of respective solvent from that of polymer solutions. The Guinier approximation eq. (7) was used to evaluate the radii of gyration of lysozyme and BSA, while the Zimm plot (the inverse of scattering intensity *vs.* q^2 plot) was employed for pullulan solutions where pullulan molecules are supposed to be randomly coiled.

RESULTS AND DISCUSSION

SAXS results are presented in terms of Kratky-plot in Figs. 1, 2, 3 and 4, respec-

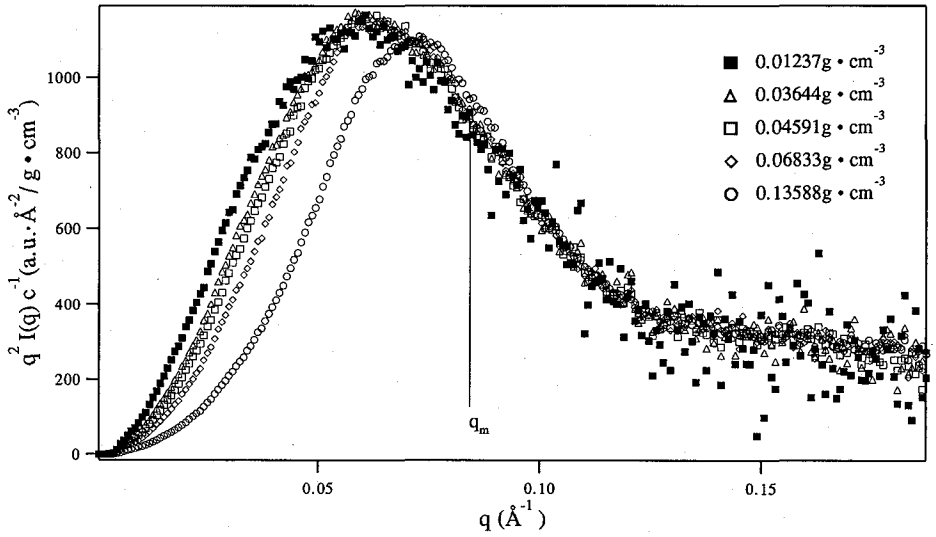

Fig. 1 Kratky-plot of SAXS from BSA solutions. Concentrations are as indicated in the figure. q_m denotes the upper integration limit, see eq. (16)

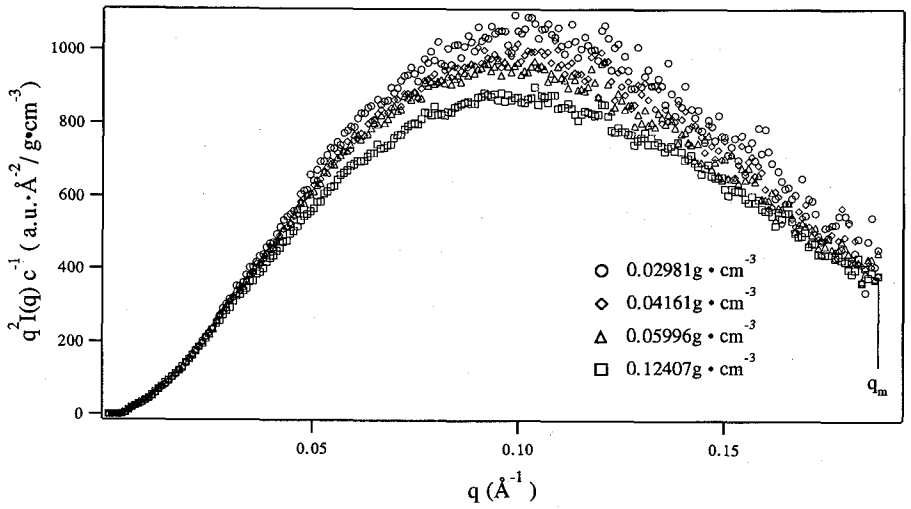

Fig. 2 Kratky-plot of SAXS from lysozyme solutions. Concentrations are as indicated in the figure. q_m denotes the upper integration limit, see eq. (16)

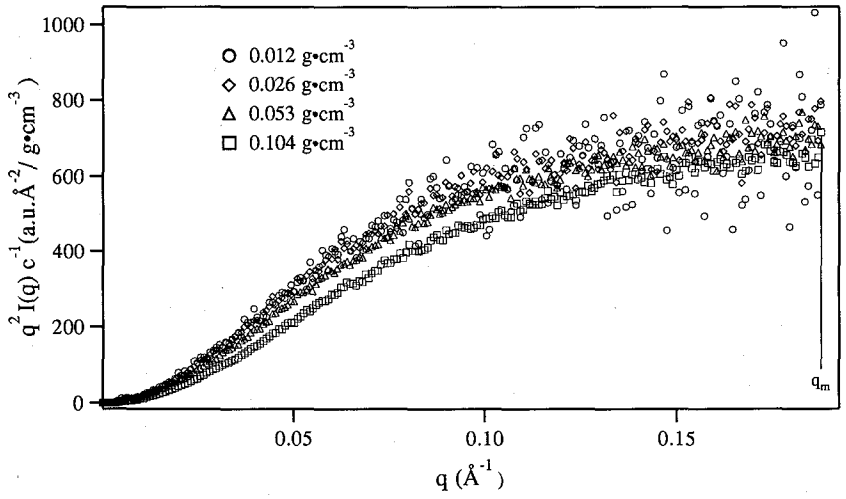

Fig. 3 Kratky-plot of SAXS from pullulan (P-5) solutions. Concentrations are as indicated in the figure. q_m denotes the upper integration limit, see eq. (16)

tively, for BSA, lysozyme and two pullulans, where each q_m was arbitrary chosen so as to satisfy eq. (14). No concentration dependence was observed as expected in scattering profiles at larger q range. This is more so in the case of BSA and lysozyme, where the q_m value can be specified uniquely, whereas pullulan exhibits rather scattered long tail in its scattering profile and q_m was taken to the largest q value in respective SAXS measurements. These scattering profiles confirm that BSA and lysozyme are rather compact in shape, while pullulan assumes expanded coil. Figs. 5, 6, 7 and 8 show the concentration dependence of (a) $I(0)$ (an extrapolated scattering intensity at zero angle) and (b) Z(an integrated scattering intensity from 0 to q_m, see

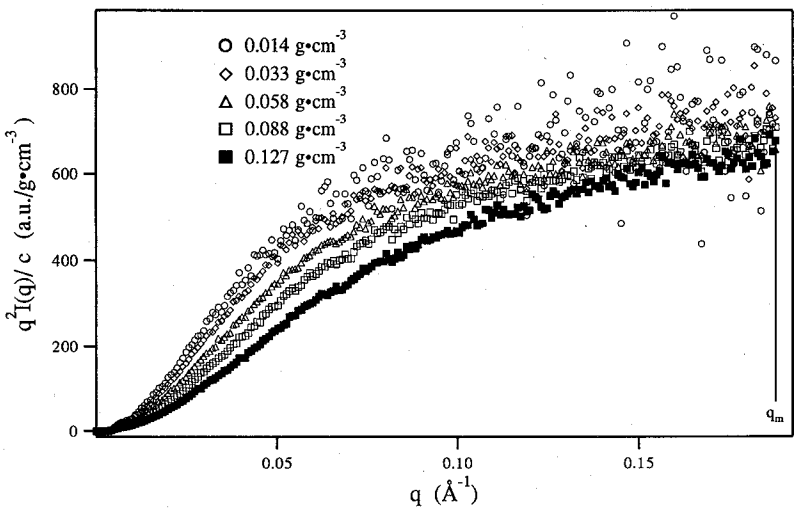

Fig. 4 Kratky-plot of SAXS from pullulan (P-10) solutions. Concentrations are as indicated in the figure. q_m denotes the upper integration limit, see eq.(16)

eq. (16)), respectively, for BSA, lysozyme and two pullulans.Here Z is defined as

$$Z \equiv \frac{1}{2\pi^2} \int_0^{q_m} \frac{I(q)}{c} \cdot q^2 \cdot dq. \tag{17}$$

Table II summarizes the extrapolated values of $I(0)$ to zero concentration and the slopes of Z vs. c plots, corresponding to the numerator and denominator in eq. (16), respectively, as well as the radius of gyration and molecular weight evaluated in the present experiments. Here the radius of gyration was evaluated from the Guinier plot for BSA and lysozyme and from the Zimm plot for pullulan.

The evaluated molecular weight 81,000 of BSA is approximately 20% larger than the value 66,100 calculated from its primary structure. Since BSA is a compact oblate in shape and its scattering profile decays sharply, the q_m value could be specified uniquely. Thus the discrepancy between estimated and calculated molecular weights is due to the present BSA sample (purchased from Sigma) containing some amounts of higher molecular weight materials as found by SDS acrylamide gel electrophoresis[6]. The radius of gyration was estimated a little smaller than the value cited in the literature[7], but lays within the experimental error. Here a good linearity was not observed in the $I(0)/c$ vs. c plots, probably because the prominent interparticular interaction makes the extrapolation of $I(q)$ to zero angle less reliable.

Lysozyme yields a reasonable agreement with the cited values[8,9] with respect to both molecular weight and radius of gyration, respectively. Since no smearing of scattering profile due to interparticular interaction was observed, the extrapolation of $I(q)$ to zero angle was performed through the Guinier approximation without any difficulty.

In the case of pullan, a big discrepancy was observed between estimated and cited

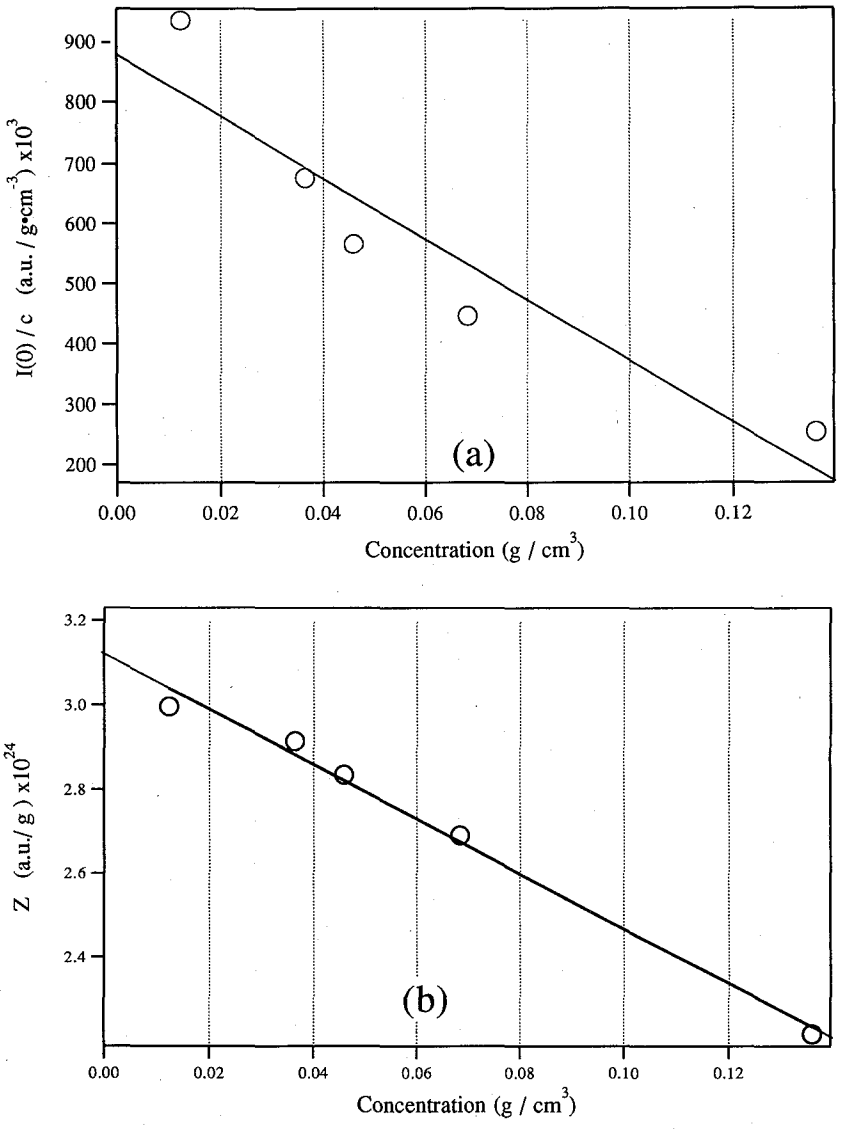

Fig. 5 Concentration dependence of (a) $I(0)/c$ and (b) Z of BSA solutions. See eq. (17) for the definition of Z.

values of molecular weights. Pullulan is expected to behave as an expanded random coil[10] when its molecular weight exceeds 2×10^4. The present samples of pullullan have a molecular weight specified as 5,800 and 12,200, respectively, and exhibits a little stiffness in its Kratky plot (Figs. 3(b) and 4(b)). The radius of gyration was estimated from the Zimm plot as 23.3 Å for P-5 (a lower molecular weight sample) and 36.0 Å for P-10 (a higher molecular weight sample), which are nearly equal to 22.9 Å and 35. 0 Å calculated from the relation[11]

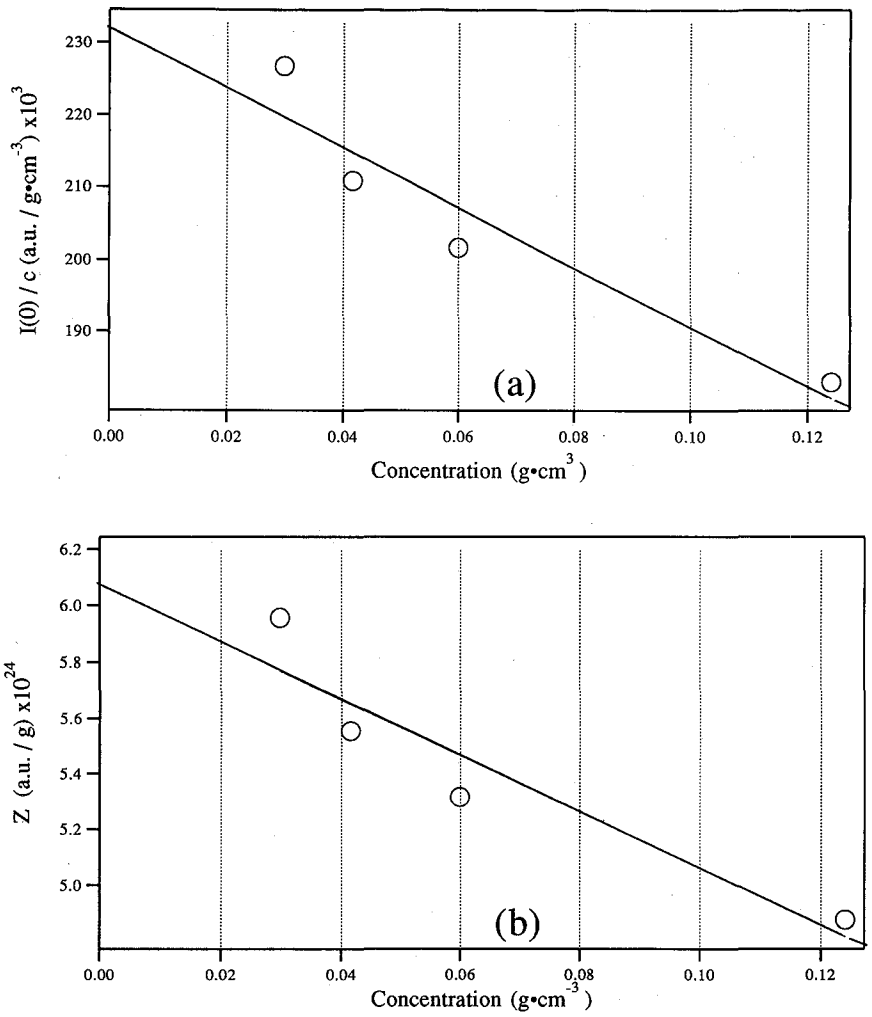

Fig. 6 Concentration dependence of (a) $I(0)/c$ and (b) Z of lysozyme solutions. See eq. (17) for the definition of Z.

$$R_G = 1.64 \times 10^{-1} M_w^{0.57}, \tag{18}$$

suggesting pullulan molecules in water to be approximated as a random coil in practice. Pullulan is reported to be stable and show no aggregation in aquesous solution[10]. However, the estimated molecular weight is nearly 100% larger for the lower molecular weight sample (P-5), or 30% larger for the higher molecular weight sample (P-10) than respective value specified by the supplier. This result may indicate an inadequacy of the present method applied to the system of not compact configuration. The error is probably due to the slow decay of the scattering profile of chain molecules, where q_m may lay in much larger range of scattering angles. Since the signal-to-noise ratio deteriorates with increasing scattering angle, no reliable q_m value

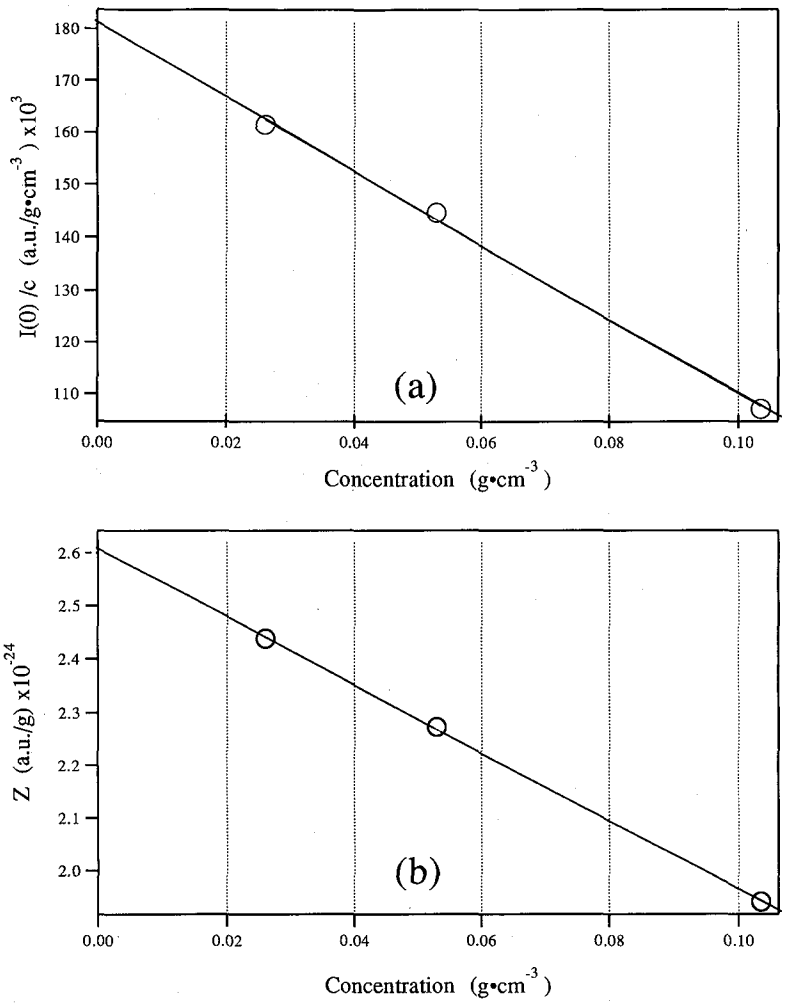

Fig. 7 Concentration dependence of (a) $I(0)/c$ and (b) Z of pullulan (P-5) solutions. See eq. (17) for the definition of Z.

can be specified, resulting a large error in estimating the molecular weight through the present method.

In conclusion, the present method allows a reliable estimation of molecular weight for compact molecules, but is inaduate to be applied to the system of random coils.

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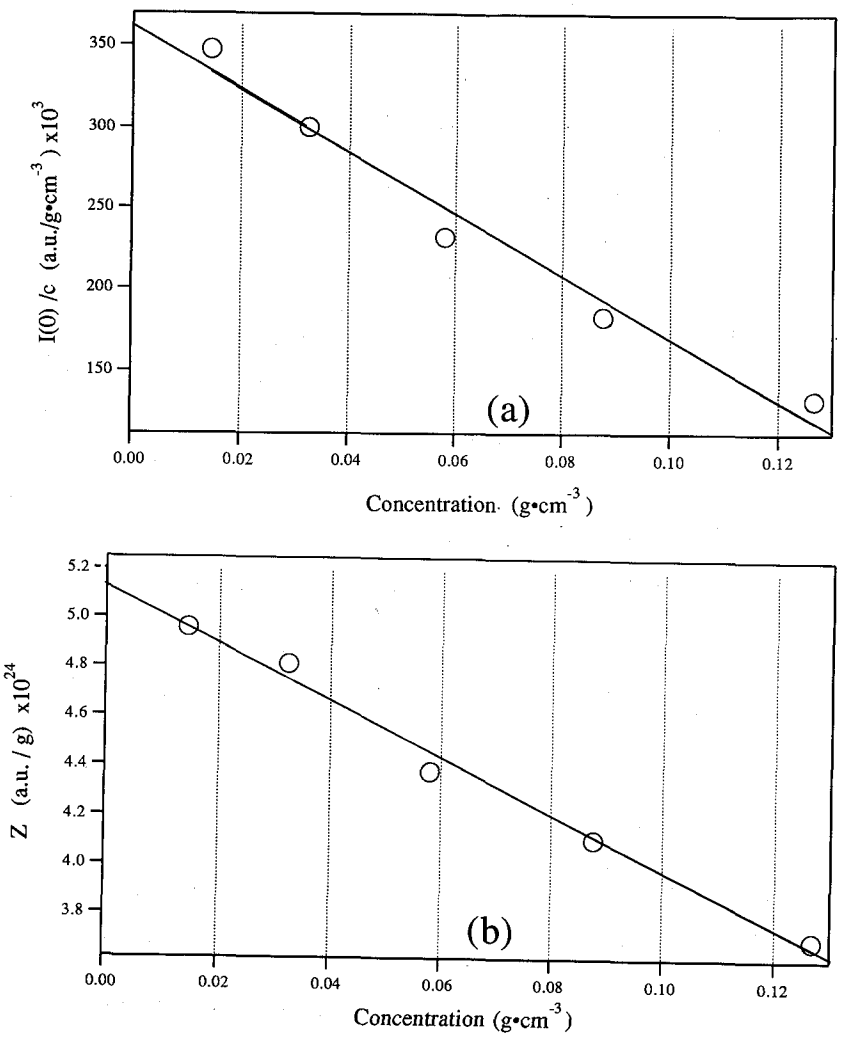

Fig. 8 Concentration dependence of (a) $I(0)/c$ and (b) Z of pullulan (P-10) solutions. See eq. (17) for the definition of Z.

Table II SAXS results

Samples	R_g (Å)	I(0)/C (Arb.units/g • cm^{-3})	q_m (Å^{-1})	dZ/dc (10^{24} × Arb.units/g^2)	Molecular Weight (g • mol^{-1})
BSA	27.7	878000	0.084	− 6.53	81,000
Lysozyme	15.2	232000	0.187	−10.09	13,800
Pululan					
P-5	23.3	180000	0.187	− 9.19	11,800
P-10	36.0	310000	0.187	−11.47	18,900

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